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I, Albrecht von Menges, Patent Attorney with UEXKÜLL AND STOLBERG, do hereby certify that I am conversant with the English and German languages and am a competent translator thereof, and I further certify that to the best of my knowledge and belief that the attached English language document is an accurate, true and correct translation made by me of German Patent Application No. 102 48 751.0, entitled 'Dyslocalization Molecules and Use Thereof' by Berdel et al., filed October 18, 2002.

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Dyslocalization Molecules and Use Thereof

The present invention relates to dyslocalization molecules, methods for their preparation and their use as medicaments, in particular for the treatment of tumors.

The localization of a protein, that is to say the location site in a cell, in a tissue or in the plasma has a substantial influence on the function and activity of the protein. This particularly applies to proteins involved in cell regulation.

Eukaryotic cells comprise intracellular membranes which divide almost one half of the cell contents into spatially separate compartments referred to as organelles. The main types of membrane-enclosed organelles occurring in all eukaryotic cells are the endoplasmic reticulum, the Golgi apparatus, the cell nucleus, the mitochondria, the lysosomes, the endosomes and the peroxisomes. Each organelle has a particular set of proteins which ensures maintenance of the organelle-specific functions.

Newly synthesized proteins make their way from the cytosol, where they are formed, to the organelle, in which they carry out specific tasks, by following a specific transport pathway. The transport pathway is defined by signals in the form of signal peptides or signal regions in the amino acid sequence of the protein. These signal peptides are recognized by corresponding receptors of the target organelle. Proteins which carry out their task in the cytosol comprise no signal peptides and therefore remain in the cytosol (Alberts et al., Molekularbiologie der Zelle; VCH Verlag, 3rd edition).

Furthermore, the targeted localization of proteins is achieved by their organization as multimeric complexes which can be specifically transported to subcellular structures. These complexes are held at appropriate sites through their affinity for anchor or scaffold proteins and by means of other structural components at this site. The affinity of individual proteins for these structures depends on the appropriate localization domains, post-translational modifications, alosteric changes and other effects (Stein et al., J.Cell.Biochem., Suppl. (2000), pp.84-92).

The function of various protein families having DNA-binding and transactivating activity, such as, for example, Catenin, Notch or STAT proteins, depends essentially on transport from the cytosol into the cell nucleus.

In many disorders, functional consequences of a mutation result in altered localization of the mutated gene products. In chronic myeloid leukemia (CML) for example, the transforming potential of Bcr-Abl depends not only on the activated kinase activity of Abl but also on the impaired, actin-bound localization of the protein. Due to this localization, both mitogenic and anti-apoptotic signal pathways are activated, resulting in the transforming activity (Daley et al., Science, Vol.247 (1990), pp.824-830).

Nuclear inclusion of Bcr-Abl by nonspecific inhibition of the nuclear export machinery leads for example to apoptosis of Bcr-Abl positive cells (Vigneri P. & Wang J.W., Nat.med., Vol.7 (2001), pp.228-234).

In acute myeloid leukemia (AML), the malignant transformation is often associated with protein dyslocalization. The most frequent chromosomal translocations generate chimeric proteins which comprise transcription factors, frequently leading to

fusion of the DNA-binding domain of a transcription activator to a transcription repressor. Thus, the transcription repressor is transported wrongly to the target genes of the transcription activator.

The most frequent chromosomal translocation in AML is the t(8;21) translocation which is found in 10-15% of adult patients afflicted with this disease (Downing J.R., Br.J. Haematol. Vol. 106 (1999), pp.296-308). Because of this translocation, the C-terminal end of the transcription activator AML1 is replaced by the transcription repressor ETO and generates the AML1-ETO fusion protein (Meyers et al., Mol.Cell.Biol., Vol.15 (1995), pp.1974-1982; and Lenny et al., Oncogene, Vol.11 (1995), 1761-1769).

The AML1-ETO fusion protein is able to effect binding of various corepressors and histone deacetylases (HDACs), and in this way to inhibit expression of the AML1 target genes, for example of GM-CSF, of neutrophil elastase and c/EBPα (Britos-Bray, M. & Friedman, A.D., Mol.Cell.Biol., Vol.17 (1997), pp.5127-5135); Frank et al., Oncogene, Vol.11 (1995), pp.2667-2674); Pabst, et al., Nat.Med., Vol.7 (2001), pp.444-451; and Oelgeschlager et al., Mol.Cell.Biol., Vol.16 (1996), pp.4717-25). It can be assumed that this effect of AML1-ETO is responsible for the AML-typical blockade of differentiation.

Hematological neoplastic diseases, such as AML, are presently treated by administration of chemotherapeutic agents. The limited availability of active drugs, specifically directed to and attacking cancer cells is a significant reason for the bad prognosis still applicable for many cancerous conditions. The therapy is always associated with serious side effects for the patient because the effect of the particular therapeutic approach affects all proliferating cells.

The side effects may lead to renal failure and organic damage caused by toxicity to the heart, lung, liver and nervous system. The consequence which must be expected from the immunosuppressant effect of this therapy is an increased number of infections leading to fatality.

Attempts have therefore been made in the prior art to develop tumor cell-specific therapeutic approaches. Thus, a deficient adenovirus able to replicate exclusively in tumors with mutations in the p53 signal transduction pathway has been constructed (Bischoff et al., 1996, Science, Vol.274, pp.373-6). By this procedure, tumor cells which have a p53 mutation are infected, whereas other cells are unaffected. The practical value of this therapy is currently being investigated in clinical trials (McCormick F., 2000, Semin.Cancer Biol., Vol.10, pp.453-9).

However, most therapeutic approaches are directed at the identification of small molecules which might be used as inhibitors of oncogenic proteins, for example specific inhibitors of tyrosine kinases. STI571, an inhibitor of various tyrosine kinases including Bcr-Abl, has proved to be effective against t(9;22) leukemias (Vigneri et al., 2001, Nat.Med., Vol.7, pp.228-34). Despite the activity of STI571 in inhibiting the molecular targets in BCR-ABL-associated disorders, full activity is achieved only in CML patients with an early (chronic phase) but not fully developed disorder. In contrast thereto, relapse is to be observed in most patients with Bcr-Abl positive acute lymphoblastic leukemia and CML blast crisis. The reason is probably that cancer is the result of a series of genetic changes, and reversal of one of these oncogenic events by an active agent is insufficient to cure the disorder.

Although molecular targets for cancer therapy are being identified at an increasing rate, scarcely any ideas

have yet been developed as to how this knowledge could be utilized for specific therapies.

The present invention was thus based on the object of providing compounds which, as an active agent of a medicament, allow for the improved treatment of tumors, in particular of leukemias.

This object has now been achieved by compounds which have binding affinity for a tumor-specific molecule and are able to effect dyslocalization of said tumor-specific molecule.

In contrast to prior art therapeutic approaches, the therapeutic approach of the present invention is thus directed at dyslocalization of an oncogenic molecule, in which the function of the oncogene is not inhibited but utilized for eliminating the oncogene-containing cells. The compounds of the invention are highly specific and have no effect whatsoever on cells which do not have the tumor-specific molecule.

In one embodiment of the present invention, the compound is a peptide, oligopeptide, protein or fusion protein. However, it is likewise possible to employ small molecules which are characterized by their specific binding to the tumor-specific molecule. A large number of organic molecules can be employed in this connection. Organic molecules mean in the present connection hydrocarbons of low molecular weight. These may have a molecular weight of <5000 Da, preferably <1000 Da and particularly preferably <500 Da. It is likewise conceivable to use composite molecules consisting of two different components.

The tumor-specific molecule is a molecule which in this form is present either exclusively in tumor cells or is present in tumor cells in a different concentration than in healthy cells. The tumor-specific molecule is

preferably also a peptide, oligopeptide, protein, fusion protein, RNA or DNA. Tumor-specific post-translational modifications such as phosphorylation, glycosylation, acetylation, methylation and similar modifications are also possible in this connection as tumor-specific parameters.

In one embodiment of the present invention, the tumor-specific molecule is a fusion protein which is present exclusively in tumor cells, for example the AML1-ETO molecule. Tumor-specific molecules which can be further attacked are the fusion proteins resulting from other chromosomal translocations in leukemias (Bcr-Abl, PML-RARalpha, PLZF-RARalpha, MLL fusion proteins, etc.) and in other malignant disorders (e.g. EWS-Fli in sarcomas).

The compounds of the invention show a binding affinity for tumor-specific molecules. The binding affinity is preferably in the range of 10^{-5} to 10^{-12}, and particularly preferably in the range of 10^{-7} to 10^{-9}.

The compounds of the invention are able to effect a dyslocalization of the tumor-specific molecules. For the purposes of the present invention, dyslocalization of a tumor-specific molecule means transport of the molecule within the cell or the tissue to a site where this molecule is not normally present in tumor cells. For example, a dyslocalization may effect a binding of tumor-specific proteins (for example transcription activators or repressors) to the genomic DNA at positions at which the tumor-specific proteins would not otherwise bind.

According to another example, dyslocalization of a tumor-specific molecule may have the result that the latter is secreted or transported into a cell organelle although it is a cytoplasmic molecule in the tumor cell. For example, the tumor-specific molecule may be

exported from the nucleus although it is a nuclear molecule in tumor cells.

According to a particularly preferred embodiment of the present invention, the dyslocation of the tumor-specific molecule leads to a more than 60% inhibition of the growth of tumor cells, wherein more than 80% inhibition is particularly preferred. The growth inhibition can be determined via a reduction in the colony formation in methylcellulose according to the method of Mizuki, M. et al. "Flt3 mutations from patients with acute myeloid leukemia induce transformation of 32D cells mediated by the Ras and STAT5 pathways", Blood, 2000 Dec 1, Vol. 96(12), 3907-14.

According to an alternative embodiment, the dyslocalization leads to an induction of apoptosis in the tumor cells. The apoptosis in the tumor cells is increased in cells treated with the molecule of the invention as compared to untreated cells preferably by a factor of 2, wherein an increase in apoptosis by a factor of at least 3 is particularly preferred. The increased induction of apoptosis in the tumor cells can be measured by means of standard assays (Darzynkiewitz, Z. et al., "Flow cytometry in analysis of cell cycle and apoptosis", Semin Hematol. 2001 Apr, Vol.38(2), 179-93).

According to the present invention, the dyslocalization of the tumor-specific molecule may lead for example to binding of the tumor-specific molecule to a nucleic acid sequence which regulates the transcription of a gene. The transcription of the gene may be activated or inhibited through the binding of the tumor-specific molecule.

According to a particularly preferred embodiment, the compound comprises the peptide sequence of the c-myb

DNA binding domain and/or the peptide sequence of the AML1 binding domain of the MEF ("myeloid elf like factor"). According to a particularly preferred embodiment, the compound of the invention has the amino acid sequence shown in SEQ ID NO:1.

The present invention further relates to nucleic acids which encode a peptide or protein according to the invention which has binding affinity for a tumor-specific molecule and can effect dyslocalization of the tumor-specific molecule. The nucleic acid is preferably DNA or RNA. The nucleic acid may be part of a vector which may be designed for expression of the nucleic acid. According to a particularly preferred embodiment, the compound of the invention is encoded by the nucleotide sequence shown in SEQ ID NO:2.

According to a further embodiment, the present invention relates to host cells which have one of the nucleic acids of the invention.

The invention further comprises medicaments which comprise a compound, nucleic acid or host cells according to the invention. The medicament may additionally comprise a pharmaceutically acceptable carrier and be formulated for oral, intravenous or intramuscular administration.

The present invention further relates to the use of the compound, nucleic acids or host cells according to the invention for the preparation of a medicament for the treatment of tumors, leukemias, especially acute myeloid leukemia. The treatment of an acute myeloid leukemia caused by a t(8;21) translocation is particularly preferred.

Methods for preparing the compounds of the invention are further included within the scope of the present invention. In the case of a peptide or protein, the

latter can be expressed recombinantly or obtained by protein synthesis.

Finally, the present invention relates to methods for identifying a compound suitable for the treatment of tumors, in which:

(a) a tumor-specific molecule is identified; and

(b) a compound which has a binding affinity for said tumor-specific molecule and is able to effect a dyslocalization of said tumor-specific molecule is identified.

Tumor-specific molecules are identified in this method by means of modern genomic and proteomic methods. It is possible to employ in this connection for example microarray analyses or 2D protein gel electrophoreses with subsequent identification by mass spectrometry and a combination of these methods.

All methods known in the art for analyzing differences between tumor cells and non-degenerated cells can be used in accordance with the invention for the identification of tumor-specific molecules.

In a second step, the target molecule which can be used for dyslocation of the tumor-specific molecule is identified. This molecule may once again be a protein, an RNA or a DNA fragment.

The screening method is preferably applied as a high-throughput method in such a way that thousands of substances are tested for their binding to the tumor-specific molecule and to the dyslocalization molecule by means of automated robotic pipettors. Subsequently, compounds which bind with high affinity and specificity to one of the two molecules or to both molecules at the same time are selected. If two different molecules are

identified (with one binding to the tumor-specific molecule and the other inducing the dyslocalization), these molecules are coupled by chemical methods, e.g. by introducing a polylinker. One great advantage of this screening method is that each molecule needs to bind only to the target molecule, but does not necessarily need also to influence the function of the target molecule.

In the following examples, a recombinant fusion protein was generated in order to direct the AML1-ETO repressor activity at promoters which are essential for the survival and the proliferation of myeloid cells. A high degree of specificity was achieved by various effects. The c-myb binding sites were used as target for GFP-M&M and AML1-ETO repressor complexes. C-myb is essential for hematopoietic cells but not for the development of other organs (Mucenski, 1991, Cell Vol.65, pp.677-89).

The essential significance of c-myb for cellular proliferations of leukemic cells is generally known. Inhibition of myb-dependent genes represents a substantial target of leukemia therapy (Mucenski, 1991, Cell Vol.65, pp.677-89; Ratajczak, 1992, Proc.Natl.Acad.Sci. USA, Vol.89, pp.118237; Gewirtz et al., 1988, Science, Vol.242, pp.1303-6; Gewirtz A.M., 1999, Oncogene, Vol.18, 3056-62).

The experiments show that the dyslocalization molecule of the invention (in this case a recombinant fusion protein) is not toxic for cells which do not express AML1-ETO. A high specific toxicity was achieved for cells which had suffered tumor-inducing transformations.

Description of the figures

Figure 1: Construction of an AML1-ETO dyslocalization protein

a Hypothesis of the function of a chimeric protein consisting of the DNA binding domain of c-myb and the AML1 binding domain of MEF.

b Structure of the chimeric protein and of the deletion mutant.

c Immunoblotting detection from Cos7 cell lysates using an anti-GFP antibody after transfection of the cells with GFP, GFP-ΔM&M and GFP-M&M.

Figure 2: Specific binding of GFP-M&M to myb binding sites and binding of AML1-ETO in vitro. Cell nuclear extracts of Cos7 cells which had been transfected with c-myb GFP-M&M and AML1-ETO were analyzed in electrophoretic mobility shift assays (EMSA). Competition experiments with specific myb and nonspecific oligonucleotides show the specificity of GFP-M&M binding. The supershift of M&M produced by cotransfection of GFP-M&M with AML1-ETO shows the dyslocalization of AML1-ETO to the myb binding sites.

Figure 3: Binding of AML1-ETO to the endogenous c-kit promoter by means of GFP-M&M. KCL22 cells were transfected with FLAG-AML1-ETO and GFP or GFP-M&M, DNA-binding proteins were firmly coupled to DNA by use of formaldehyde, the cells were lyzed, and the DNA was fragmented and immunoprecipitated with anti-FLAG or nonspecific antibodies. A PCR was used to detect the promoter sequences of c-kit and $p14^{ARF}$ in the immunoprecipitated chromatin. One representative of two experiments is shown.

Figure 4: Specific repression of the myb-dependent promoter by GFP-M&M in the presence of AML1-ETO. KCL22 cells were transiently transfected with a myb-dependent luciferase construct and c-myb, AML1, AML1-ETO, GFP-ΔM&M and GFP-M&M (as indicated). The mean value and the standard error of three independent experiments is depicted.

Figure 5: GFP-M&M represses colony growth in AML1-ETO expressing cells.

a 32D cells were transfected with GFP as control or AML1-ETO and GFP-M&M as indicated, and 1×10^5 cells were seeded in colony detection methods. The photographs show representative colonies on day 10.

b 32D cells were transfected as indicated and seeded in the colony detection method. The colonies were counted on day 10. The repression of colony growth compared with the control transfection with GFP (set equal to 1) is depicted here. The mean value and the standard error of three independent experiments is shown.

c AML1-ETO was transfected alone or in combination with GFP-M&M or GFP-ΔM&M into 32D cells and then seeded for colony detection methods. The mean value and the standard error of three independent experiments is depicted.

d GFP or GFP-M&M were transfected into Kasumi-1 cells which naturally express AML1-ETO, and seeded in colony detection methods. The mean value and the standard

error of three independent experiments is depicted.

Figure 6: GFP-M&M induces apoptotis in AML1-ETO expressing cells. 32D cells were transfected with AML1-ETO, GFP-M&M or both vectors and then the transfected cells were sorted by flow cytometry. The transfected cells were then analyzed in a TUNEL detection method.

a Representation of the FACS results for BrdU-positive apoptotic cells. The unshaded curves represent the apoptosis rate in the cells transfected with a empty vector for control purposes.

b Representation of the proportions of apoptotic cells in the transfected 32D cells.

Material and methods

The following material and methods were used in the examples:

1. **Plasmids:**

The GFP-M&M expression plasmid in pcDNA3.1 was prepared by means of a PFU polymerase chain reaction (PCR) using a murine c-myb expression plasmid and cDNA from KCL22 cells as template, wherein specific primers for the DNA binding domain of c-myb (encoded by nucleotides 193-594 of SEQ ID NO:13; including the restriction sites for KpnI and BamHI) and the AML1 binding domain of MEF (encoded by nucleotides 251-618 of SEQ ID:12; including restriction sites for BamHI and EcoRI) were used. The PCR products were cloned in reading direction into GFP-pcDNA3.1 (GFP corresponds to nucleotides 91-813 of SEQ ID NO:11). GFPΔM&M was

cloned correspondingly, using a PCR fragment which lacked the first 159 base pairs of the DNA binding domain of c-Myb.

<u>Primers for the AML1 binding domain of MEF:</u>

MEF-BamH1 for:5'- ATA GGA TCC GCC ACC TCG CAC ACC ATG TCA-3' (SEQ ID NO: 3)
MEF-EcoR1 rev:5'- CAG AAT TCG CCT TTG CCA TCC TTT GAT TTC-3' (SEQ ID NO: 4)

<u>Primers for the DNA binding domain of c-myb:</u>

myb-Kpn1 for: 5'- CAG AGA GGT ACC GTC ATT GCC AAT TAT CTG-3' (SEQ ID NO: 5)
myb-BamH1 rev:5'- CAG AGA GGA TCC GTA GCC TTC CTG TTC CAC-3' (SEQ ID NO: 6)

The myb-TK (thymidine kinase) luciferase construct was a gift from Prof. Klempnauer. The AML1-ETO cDNA was subcloned into pcDNA3.1.

2. **Cell lines and transfection**:
The IL-3-dependent murine myeloid cell line 32Dcl3, the human myeloid cell lines KCl22 and Kasumi-1, and the monkey kidney cell line Cos7 were cultured by methods known in the art. 32Dcl3 cells and KCl22 cells were transfected with 15 µg of plasmid DNA by electroporation, and Cos cells were transfected with 5 µg of plasmid DMA using Lipofectamine (Invitrogen).

3. **Immunoblotting**:
Protein lysates were prepared from the Cos cells transfected with the expression vectors for GFP, GFP-M&M or GFP-ΔM&M. The three proteins were detected using the monoclonal murine GFP antibody (Clonetech, Heidelberg, Germany), detection taking place through incubation with radish peroxidase

conjugated secondary IgG antibodies against mouse IgG (Jackson ImmunoResearch).

4. **Electrophoretic mobility shift assay**

Cos7 cells were transfected with a total amount of 5 μg of the expression vectors for c-myb, AML1-ETO, GFP and GFP-M&M in various combinations. The preparation of cell nuclear extracts of the transfected Cos7 cells, the binding reaction and the oligonucleotides which have the c-myb consensus binding sequence are described in Müller et al., 1999, Blood, Vol.94, pp. 4255-62. 100 ng of double-stranded oligonucleotides which had either the myb consensus site or a nonspecific binding site were used for the competitive experiments.

5. **Chromatin immunoprecipitation:**

KCL22 cells were transfected with FLAG AML1-ETO and GFP or GFP-M&M. 12 hours after the transfection, the cellular proteins were bound to the DNA by adding 1% formaldehyde for 10 minutes, and then the reaction was stopped by adding 0.125 M glycine. The cells were washed twice in ice-cold PBS and lyzed in 1 ml of RIPA lysis buffer with protease inhibitors, 200 μM sodium orthovanadate and 50 μM NaF. After incubation on ice for 10 minutes, the chromatin was fragmented by use of UV rays (9 pulses of 5 seconds). The cell debris was removed by centrifugation, and 50 μl were stored as input control. The remainder of the lysate was prepurified in 40 μl of protein A/G agarose with 5 μg of rabbit and mouse IgG. The remainder of each lysate was divided into two samples, and the immunoprecipitation was carried out either using 3 μg of an anti-FLAG or mouse IgG with 40 μl of protein A/G agarose overnight. The immunocomplexes were washed eight times in a buffer with a low salt content (0.1% SDS, 150 μM

NaCl, 1% Triton X-100, 2 μM EDTA, pH 8.0, 20 μM Tris-HCl, pH 8.1). The connections between the DNA and the proteins in the immune complexes and the input control were then redissolved, and the DNA was phenol/chloroform extracted from the solution. Subsequently, specific promoter sequences for the c-kit promoter region and the $p14^{ARF}$ promoter region were detected in the samples by means of PCR.

The PCR was carried out with a Taq polymerase (Promega) on a Mastercycler (Eppendorf) (95°C for 3 min., 37 cycles at 95°C for 1 min., 60°C for 1 min. and 72°C for 1 min.). The products were separated on a 2% agarose gel and stained with ethidium bromide.

<u>Primers for the $p14^{ARF}$ promoter region:</u>

$p14^{ARF}$ for: 5'-AGT GGC TAC GTA AGA GTG ATC GC-3' (SEQ ID NO: 7)

$p14^{ARF}$ rev: 5'-CTT ACA GAT CAG ACG TCA AGC CC -3' (SEQ ID NO: 8)

<u>Primers for the c-kit promoter region:</u>

c-kit for: 5'- ACT GTT GTT GCT TTC CGT TCA A-3' (SEQ ID NO: 9)

c-kit rev: 5'- TTA AGC CCG ATT TCA CTG CC-3' (SEQ ID NO: 10)

6. **Luciferase detection:**

The promoter activity was detected by methods known in the art (Müller et al., 2000, Mol.Cell.Biol., Vol.20, pp. 3316-29). This entails a total amount of 15.5 μg of plasmid being transfected by electrophoresis. The mixture consisted of 5 μg of a myb-TK luciferase construct, 0.5 μg of PRL-null plasmid (Promega, Madison, WI) for internal standardization and 5 μg of the expression vectors for AML1, AML1-ETO, GFP-

ΔM&M and GFP-M&M in various combinations. Empty vector was cotransfected to equalize the total amount of transfected DNA. After 18 hours, the cells were lyzed, and the activity of the firefly and renilla luciferase was detected using the dual luciferase assay system from Promega. The renilla luciferase activity was used for internal standardization of the transfection efficiency. The mean values and standard errors were calculated from three independent experiments.

7. **Clonal growth in methylcellulose:**
32Dcl3 cells and Kasumi cells were transiently transfected with a total amount of 15 g of the expression vectors for AML1, AML1-ETO, GFP, GFP-M&M and GFP M&M in various combinations. To investigate the clonal growth, the transfected cells were separated on the day following the electroporation by gradient centrifugation, and seeded in a concentration of 1×10^5 live cells per 35 mm plate in 1 ml of a culture mix. This mix consisted of Isocove modified Dulbecco medium (IMDM, Life Technologies, Grand Island, N.Y.), 1% methylcellulose, 20% FCS, IL-3 (1 ng/ml) and 0.6 mg/dl G418. All experiments were set up in triplicate, and the colonies were counted on day 10. Mean values and standard errors were calculated from three independent experiments (two experiments for Kasumi cells).

8. **Apoptosis assay:**
32Dcl3 cells were transiently transfected with the expression vectors for GFP, GFP-M&M and AML1-ETO in various combinations. After 24 hours, the GFP-positive cells were sorted out from the total cells by flow cytometry, and investigated further. The percentage of apoptotic cells among the GFP-positive cells was determined using a TUNEL assay (APO-BrdU kit from PharMingen), the experiments

being carried out in accordance with the manufacturer's instructions. The results of one of these three independent experiments with similar results are shown.

<u>Example 1</u> <u>Cloning of GFP-M&M and expression in Cos cells</u>

A fusion protein composed of the enhanced green fluorescent protein (GFP, for detection purposes; encoded by nucleotides 91-813 of SEQ ID NO:11), of the DNA binding domain of murine c-myb (nucleotides 193-594 of SEQ ID NO:13 encode amino acid residues 65-198 of murine c-myb; cf. Sakura et al., 1989, Proc.Natl.Acad.Sci. USA, Vol.86, pp. 5758-61) and of the AML1-binding domain of the human myeloid elf like factor, MEF (nucleotides 251-618 of SEQ ID:12 encode amino acid residues 87-206 of human MEF; cf. Mao S. et al., 1999, Mol.Cell.Biol., Vol.19, pp.3635-44)) was constructed.

The transcription factor c-myb is known to be essential for normal hematopoiesis and the survival of hematopoietic cells (Mucenski et al., 1991, Cell, Vol.65, pp.677-89). It was shown that inhibition of c-myb by antisense strategies or c-myb knock out mice are unable to develop normal hematopoiesis (Ratajczak et al., 1992, Proc.Natl.Acad.Sci. USA, Vol.89, pp.11823-7).

The AML1 binding domain of MEF (myeloid like ELF factor) was used as second part of the chimeric protein. Amino acids 87-206 of MEF bind strongly to AML1 and AML1-ETO in vivo and in vitro (Mao S. et al., 1999, Mol.Cell.Biol., Vol.19, pp.3635-44). All three domains were cloned in reading direction into the expression vector pcDNA3.1. This construct was referred to as GFP-M&M (Figure 1b). A deletion mutant lacking the first 53 amino acids of the DNA binding domain of

c-myb was prepared for control purposes. The deletion mutant was referred to as GFP- M&M.

Expression of the recombinant proteins was analyzed after transient transfection in Cos7 cells by means of immunoblotting detection methods using anti-GFP antibody (GFP alone 35 kDa, GFP- M&M 80 kDa; GFP-M&M 85 kDa; Fig. 1c).

Example 2 Analysis of the binding of GFP-M&M to myb binding sites

Electrophoretic mobility shift assays were carried out to analyze the interaction of GFP-M&M with myb DNA binding sites (cf. Figure 2). For this purpose, nuclear extracts of transiently transfected Cos7 cells were prepared. A double-stranded myb consensus oligonucleotide served as target DNA. These experiments showed that GFP-M&M, like c-myb, specifically binds to myb DNA binding sites. AML1-ETO alone showed no binding to the myb binding sites, but GFP-M&M led to binding of AML1-ETO to the DNA. This resulted in a supershift of the complex consisting of DNA, GFP-M&M and AML1-ETO (Fig.2).

Example 3 GFP-M&M binds AML1-ETO to the endogenous c-kit promoter

Binding of GFP-M&M and binding of AML1-ETO to endogenous c-myb target promoters was analyzed using a chromatin immunoprecipitation detection method (ChiP) in KCL22 cells.

The c-kit promoter was chosen as c-myb dependent endogenous promoter. The demonstrated binding of AML1-ETO to the $p14^{ARF}$ promoter (Linggi et al., Nature Medicine, 8 (7), July 2002) was analyzed as positive control. A FLAG-labeled form of AML1-ETO was expressed in combination with GFP or GFP-M&M in KCL22 cells. The

transcription factors were crosslinked with DNA by using formaldehyde. After cell lysis and DNA fragmentation, the DNA/AML1-ETO complexes were immunoprecipitated by using an anti-FLAG antibody or, for control purposes, nonspecific antibodies. The crosslinking was abolished and the presence of the c-kit and $p14^{ARF}$ promoter DNA sequences was analyzed by PCR. The sequence of the c-kit promoter was undetectable in the ChiP samples of the KCL22 cells transfected with AML-ETO and GFP.

However, in the presence of GFP-M&M, the c-kit promoter sequences were immunoprecipitated with AML1-ETO (Fig.3). In contrast thereto, the $p14^{ARF}$ promoter sequence was detected in immune complexes from KCL22 cells transfected with AML1-ETO and GFP. The sequence was not detected in the presence of AML1-ETO and GFP-M&M (Fig.3).

These results show that GFP-M&M is able to bind AML1-ETO to the endogenous c-myb dependent promoter c-kit in vivo.

Example 4 Inhibition of myb dependent promoters in the presence of GFP-M&M and AML1-ETO

GFP-M&M binds to myb dependent promoters, forms a complex with AML1-ETO (where present) and thus inhibits gene expression.

Luciferase assays were carried out as a further demonstration, the luciferase gene being under the control of a minimal thymidine kinase promoter with three additional myb DNA binding sites (Ziebold et al., 1997, Curr.Biol., Vol.7, pp. 253-60). KCL22 cells were transfected with the reporter constructs and GFP, GFP-M&M and AML1-ETO in various combinations (Fig.4). None of the proteins was able on its own to have a substantial influence on luciferase activity. Cells

expressing GFP-M&M and AML1-ETO together did, however, show a more than 5-fold inhibition of promoter activity (Fig.4). It was then analyzed whether the functional interaction between GFP-M&M and myb DNA binding sites was necessary for the inhibition of the luciferase activity by GFP-M&M in AML1-ETO positive cells. The mutation of the DNA binding site in GFP-ΔM&M inhibits the DNA binding of the recombinant protein, although expression of the protein is unchanged (Figure 1b). Neither expression of GFP-ΔM&M alone nor expression of GFP-ΔM&M and AML1-ETO together inhibited luciferase activity.

These results show that GFP-M&M binding to the DNA is necessary for repression of the myb dependent gene in the presence of AML1-ETO (Fig.4).

Example 5 Inhibition of colony growth by GFP-M&M in AML1-ETO expressing cells

The activity of the transcription factor Myb and the expression of the myb dependent genes are essential for the growth and proliferation of hematopoietic cells (White et al., 2000, Oncogene, Vol.19, pp.1196-205). Therefore, the effect of GFP-M&M on the proliferation and survival rate of AML1-ETO-containing cells was analyzed.

Firstly, the ability of the transfected hematopoietic 32D cells to form colonies was investigated. Colony growth was not inhibited in cells transfected with AML1, GFP-M&M or AML1 and GFP-M&M.

Cells transfected with AML1-ETO alone showed a six-fold inhibition of colony growth, which is probably attributable to the toxic effect of AML1-ETO itself (Muller et al., Mol.Cell.Bio., 2000, Vol.20., pp.3316-29). Transfection of the 32D cells with GFP-M&M in the presence of AML1-ETO did, however, reduce the growth of

the colonies about 60-fold (Figure 5a and 5b). In comparison to GFP-M&M and GFP-ΔM&M in colony assays it was found that coexpression of GFP-M&M and AML1-ETO reduced the relative number of colonies by more than 80%. In contrast thereto, GFP-ΔM&M and AML1-ETO did not inhibit colony growth (Fig.5c). The latter observation shows that a functional interaction between GFP-M&M and myb DNA binding sites is necessary for inhibition of colony growth in AML-ETO positive cells.

Besides the effects of GFP-M&M in AML1-ETO transfected cells, the activity of GRP-M&M in t(8;21) positive Kasumi-1 leukemia cells was also investigated. The colony growth was reduced twelve-fold (Fig.5d) after transfection with GFP-M&M as compared to cells transfected with GFP-pcDNA3.1 alone (control).

<u>Example 6</u> <u>Induction of apoptosis by GFP-M&M in AML1-ETO-containing cells</u>

Hematopoietic cells having no c-myb activity are subject to apoptosis (Taylor et al., 1996, Genes Dev., Vol10, pp.2732-44).

In order to investigate the effect of GFP-M&M in AML1-ETO positive cells and its influence on apoptosis, the presence of DNA strand breaks was investigated by a TUNEL assay. 32D cells were transfected with GFP, AML1-ETO or GFP-M&M or with a combination of AML1-ETO and GFP-M&M. After 24 hours, about 10% of the cells which expressed GFP, AML1-ETO or GFP-M&M alone were undergoing apoptosis. In contrast thereto, the percentage of apoptotic cells among the cells which expressed both AML1-ETO and GFP-M&M was 39%. This corresponds to a four-fold increase in the apoptosis rate (Fig.6a and b).

Sequence listing

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cactgggcag agaacagcct gagcacccaa ctgttctcgc aggcgtctcc tgtggcagat 1680
gccccaaata ttcttacaag ctctgtttta atgacacctg tatcagaaga tgaagacaat 1740
gtcctcaaag cctttaccgt acctaagaac aggcccctgg tgggtccctt gcagccatgc 1800
agtggtgcct gggagccagc atcctgtggg aagacagagg accagatgac ggcctccggt 1860
ccggctcgga aatacgtgaa cgcgttctca gctcgaactc tggtcatgtg aga 1913

Claims

1. A compound which has binding affinity for a tumor-specific molecule and is able to effect dyslocalization of the tumor-specific molecule.

2. The compound of claim 1, which is a peptide, oligopeptide, protein, fusion protein, or an organic molecule having a molecular weight of < 5000, < 1000 or < 500.

3. The compound of any of claims 1 oder 2, in which the tumor-specific molecule is a peptide, oligopeptide, protein, fusion protein, RNA or DNA.

4. The compound of any of claims 1 to 3, which has a binding affinity of 10^{-5} to 10^{-12}, and particularly preferably of 10^{-7} to 10^{-9}.

5. The compound of any of claims 1 to 4, in which the tumor-specific molecule is not present in healthy cells or is present in another form.

6. The compound of any of claims 1 to 5, in which the tumor-specific molecule is a fusion protein.

7. The compound of any of claims 1 to 6, in which the tumor-specific molecule is AML1-ETO.

8. The compound of any of claims 1 to 7, in which the tumor-specific molecule has a DNA binding domain, a signal peptide, kinase activity, chromatin-modulatory properties, protein-protein interaction domains or transcriptional properties.

9. The compound of any of claims 1 to 8, in which the dyslocalization inhibits the growth of tumor-specific cells.

10. The compound of any of claims 1 to 9, in which the dyslocalization induces apoptosis in tumor-specific cells.

11. The compound of any of claims 1 to 10, in which the dyslocalization binds the tumor-specific molecule to a nucleic acid sequence which regulates the transcription of a gene.

12. The compound of any of claims 1 to 11, in which the dyslocalization binds the tumor-specific molecule to a nucleic acid sequence which regulates the transcription of a gene, thereby activating or inhibiting the transcription of the gene.

13. The compound of any of claims 1 to 12, in which the compound comprises the peptide sequence of the c-myb DNA binding domain.

14. The compound of any of claims 1 to 13, in which the compound comprises the peptide sequence of the AML-1 binding domain of the myeloid elf like factor.

15. The compound of claim 13 or 14, in which the compound comprises the peptide sequence of the c-myb DNA binding domain and the peptide sequence of the AML-1 binding domain of the myeloid elf like factor.

16. The compound of claim 15, in which the compound has the sequence shown in SEQ ID NO: 1.

17. A nucleic acid encoding a peptide or protein of any of claims 2 to 16.

18. The nucleic acid of claim 17, which is a DNA or RNA.

19. A vector comprising a nucleic acid of claim 17 or 18.

20. A host cell having a nucleic acid of claim 17 or 18 or a vector of claim 19.

21. A medicament comprising a compound of any of claims 1 to 16, a nucleic acid of claim 17 or 18, a vector of claim 19 or a host cell of claim 20.

22. The medicament of claim 21, which further comprises a pharmaceutically acceptable carrier.

23. The medicament of claim 21 or 22, which is formulated for oral, intravenous or intramuscular administration.

24. The use of a compound of any of claims 1 to 16, a nucleic acid of claim 17 or 18, a vector of claim 19 or of a host cell of claim 20 for the preparation of a medicament for the treatment of tumors.

25. The use of claim 24, wherein the tumor is leukemia, in particular acute myeloid leukemia.

26. A method for the preparation of a compound of any of claims 1 to 16, in which the peptide or protein is recombinantly expressed or obtained by protein synthesis.

27. A method for identifying a compound suitable for the treatment of tumors, in which:

(a) a tumor-specific molecule is identified;

(b) a compound which has a binding affinity for said tumor-specific molecule and is able to effect a dyslocalization of said tumor-specific molecule is identified.

28. The method of claim 27, in which the tumor-specific molecule is identified by microarray analyses, 2D protein gel electrophoreses with subsequent identification by mass spectrometry, or a combination of said methods.

29. The method of any of claims 27 or 28, in which the compound which has a binding affinity for the tumor-specific molecule and is able to effect a dyslocalization of the tumor-specific molecule is a protein, an RNA, a DNA or an organic compound.

30. The method of any of claims 27 to 29, in which the compound which has a binding affinity for the tumor-specific molecule and is able to effect a dyslocalization of the tumor-specific molecule is identified by means of high-throughput screening methods.

31. The method of any of claims 27 to 29, in which the compound which has a binding affinity for the tumor-specific molecule and is able to effect a dyslocalization of the tumor-specific molecule has been constructed from two parts.

32. The method of claim 31, in which one part of the compound has a binding affinity for the tumor-specific molecule, and the second part is able to effect the dyslocalization of the tumor-specific molecule.

33. The method of claim 31 or 32, in which the two parts are identified in separate screening methods.

34. A method for the preparation of a medicament, comprising the steps of:

    (a) identifying a compound suitable for the treatment of tumors by a method of claims 27 to 33;
    (b) preparing the compound by synthesis or recombinantly; and
    (c) formulating the compound to give a medicament.

35. The method of claim 34, wherein the medicament is suitable for the treatment of tumors, for example for the treatment of leukemia and in particular for the treatment of acute myeloid leukemia.

FIGURE 1A

FIGUR 1A

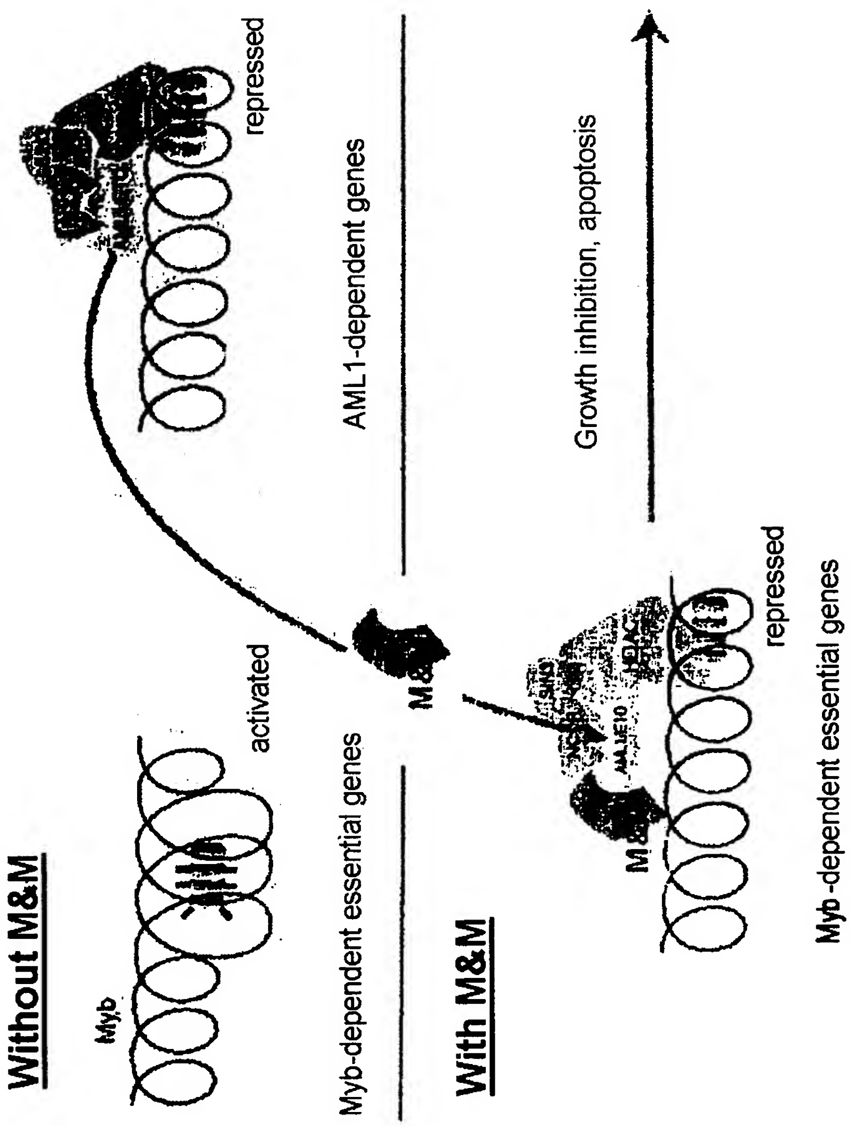


FIGURE 1B

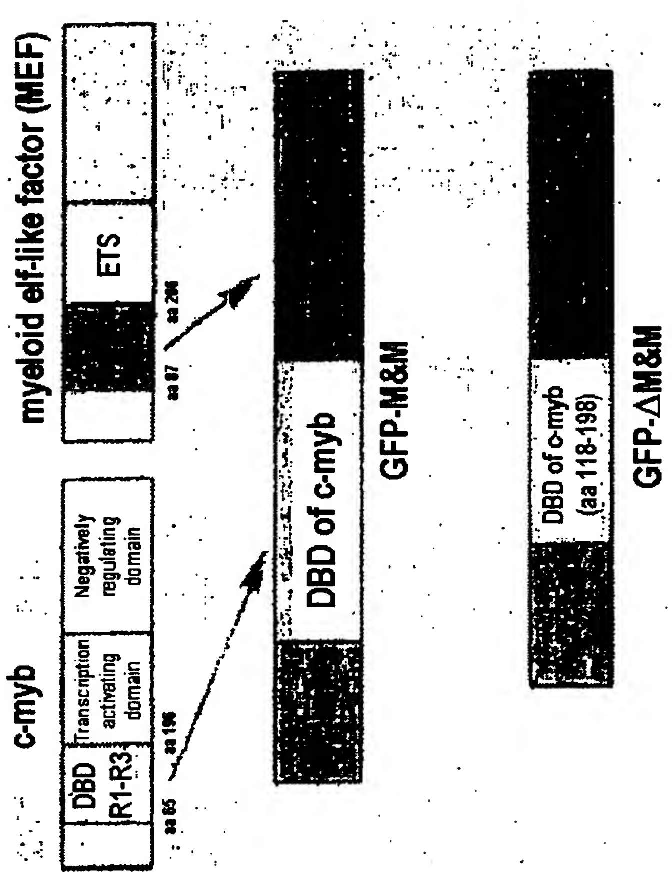

c-myb
DBD
R1-R3
Transcription activating domain
Negatively regulating domain
myeloid elf-like factor (MEF)
ETS
DBD of c-myb
GFP-M&M
DBD of c-myb
(aa 118-198)
GFP-ΔM&M

FIGURE 1C

FIGURE 2

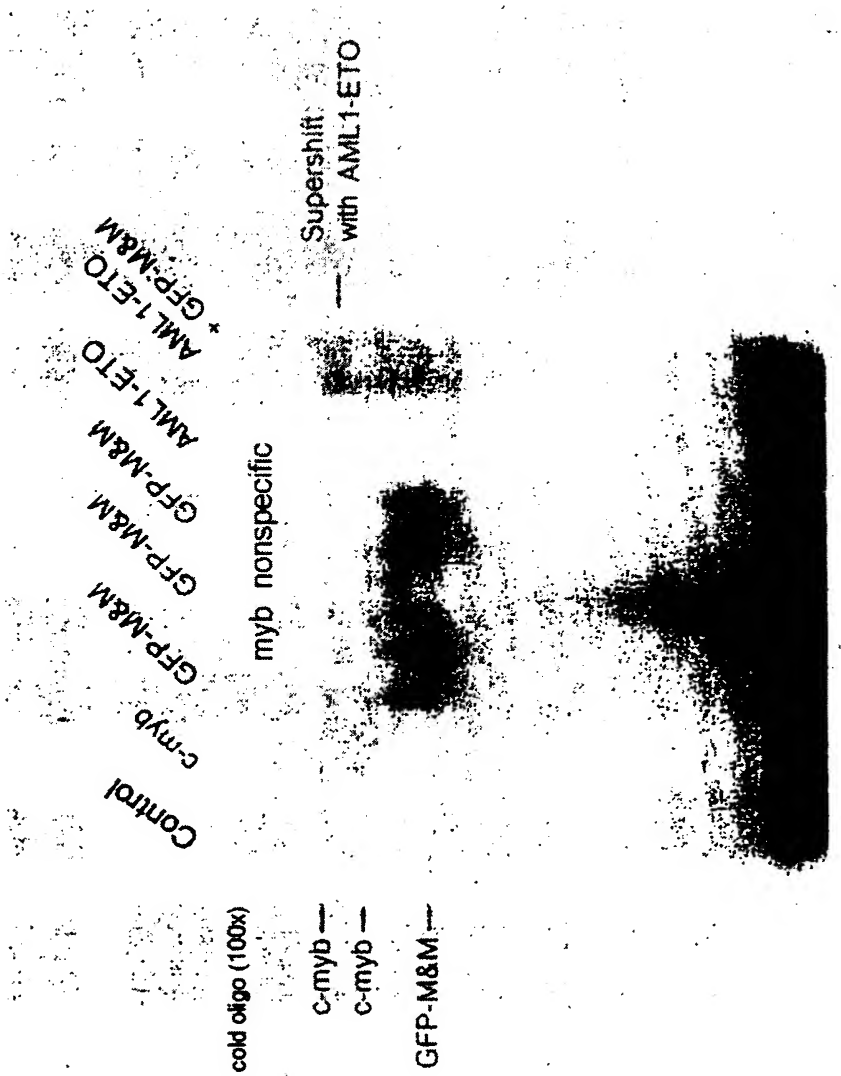

Control
c-myb
GFP-M&M
GFP-M&M
GFP-M&M
AML1-ETO
AML1-ETO + GFP-M&M
myb nonspecific
Supershift with AML1-ETO
cold oligo (100x)
c-myb
c-myb
GFP-M&M

FIGURE 3

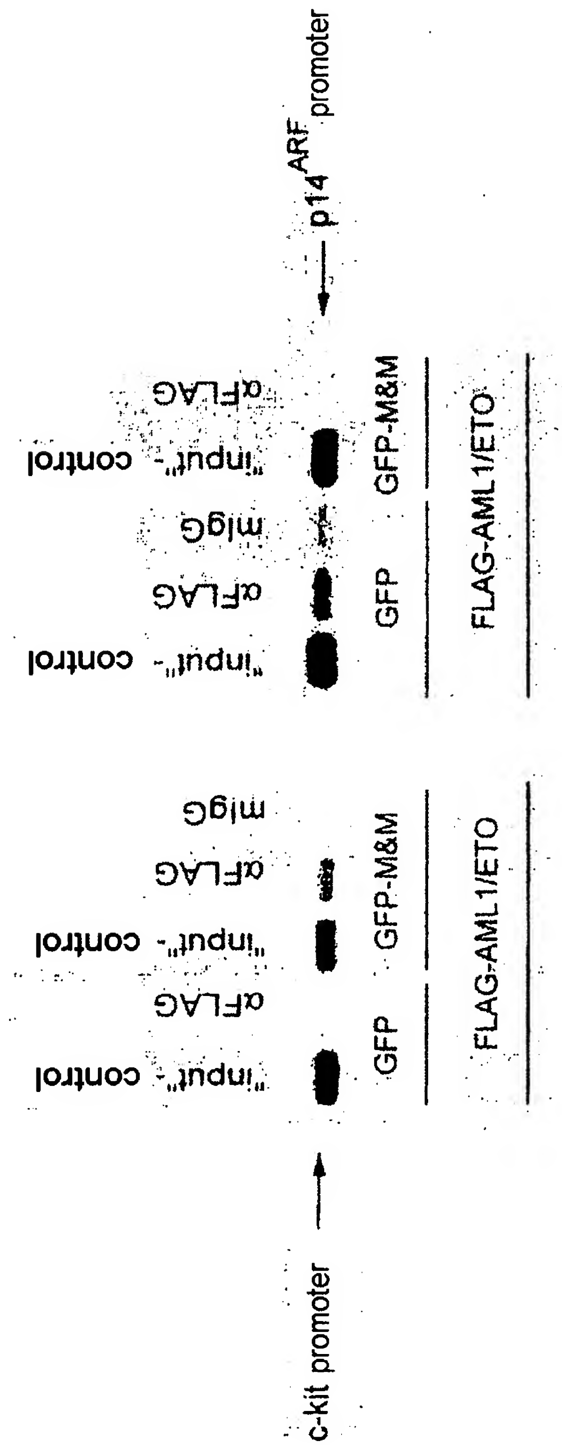
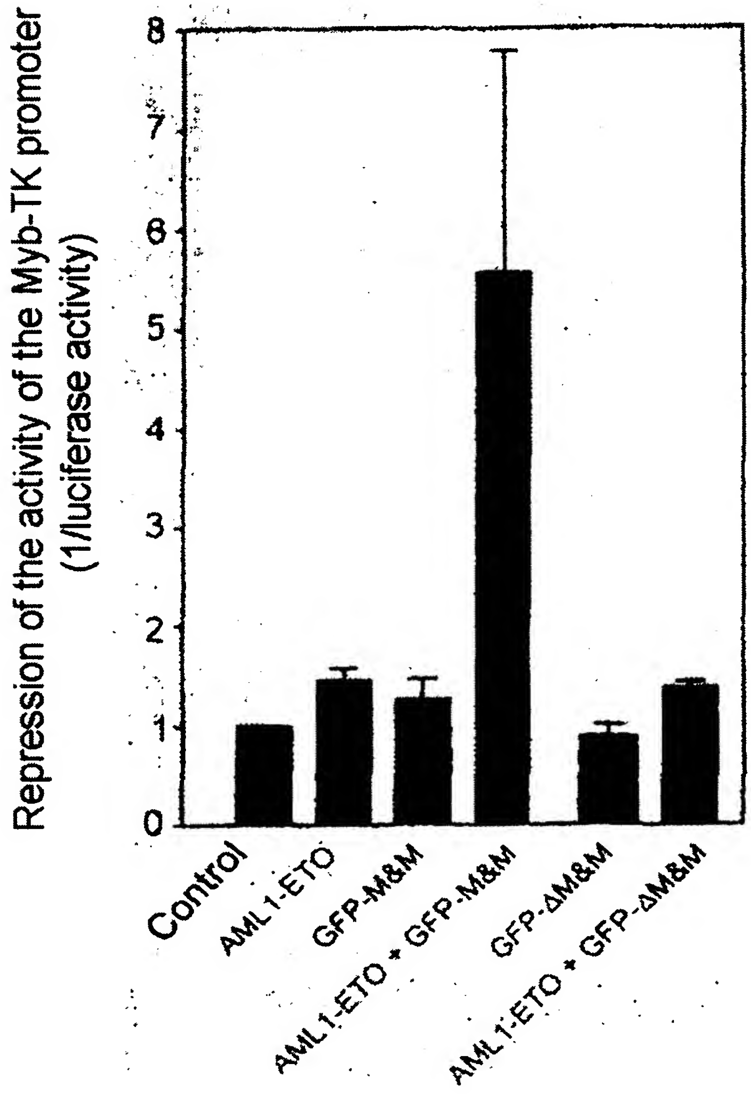

p14ARF promoter
c-kit promoter
"input"- control
αFLAG
mIgG
GFP
GFP-M&M
FLAG-AML1/ETO

FIGURE 4



Repression of the activity of the Myb-TK promoter
(1/luciferase activity)
0
1
2
3
4
5
6
7
8
Control
AML1-ETO
GFP-M&M
AML1-ETO + GFP-M&M
GFP-ΔM&M
AML1-ETO + GFP-ΔM&M

FIGURE 5A

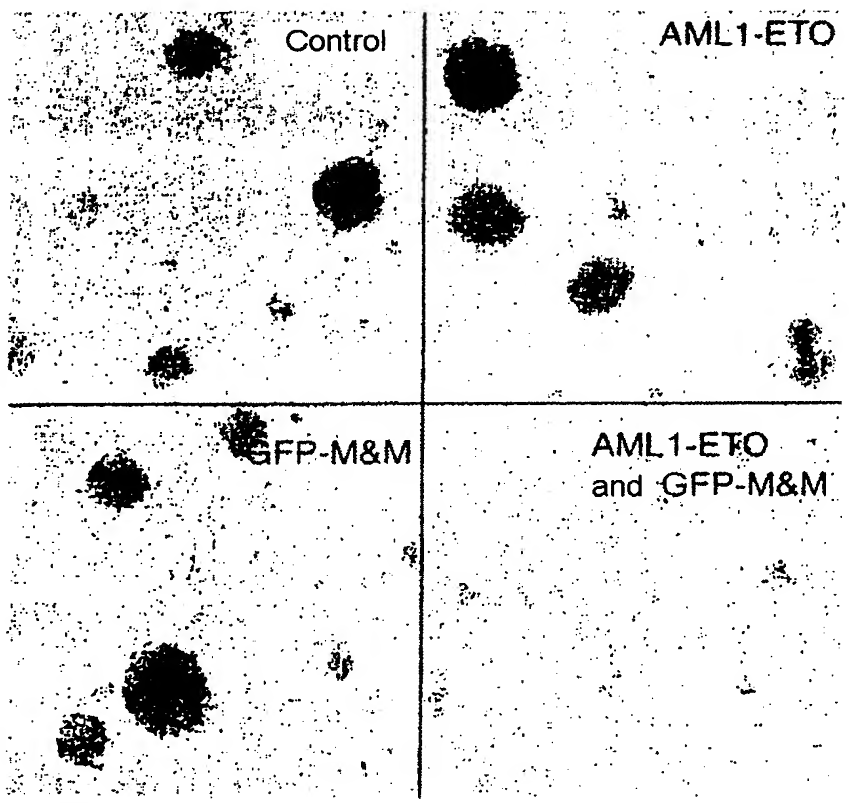


FIGURE 5B

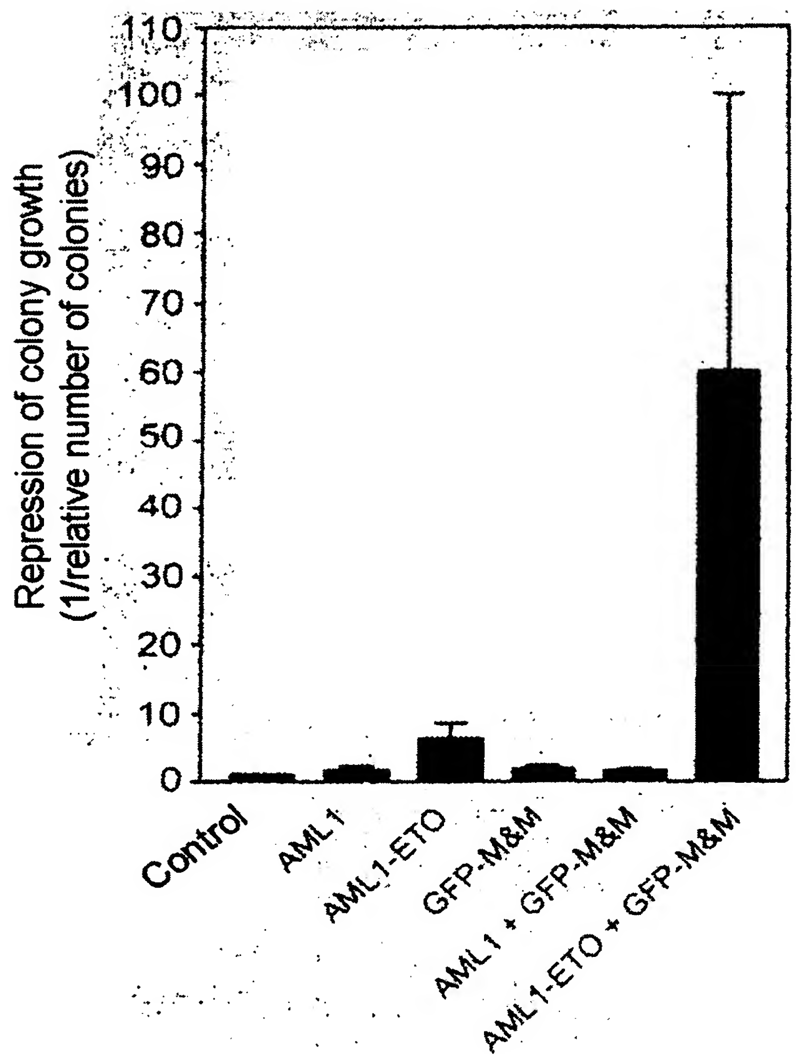

Repression of colony growth
(1/relative number of colonies)
110
100
90
80
70
60
50
40
30
20
10
0
Control
AML1
AML1-ETO
GFP-M&M
AML1 + GFP-M&M
AML1-ETO + GFP-M&M

FIGURE 5C

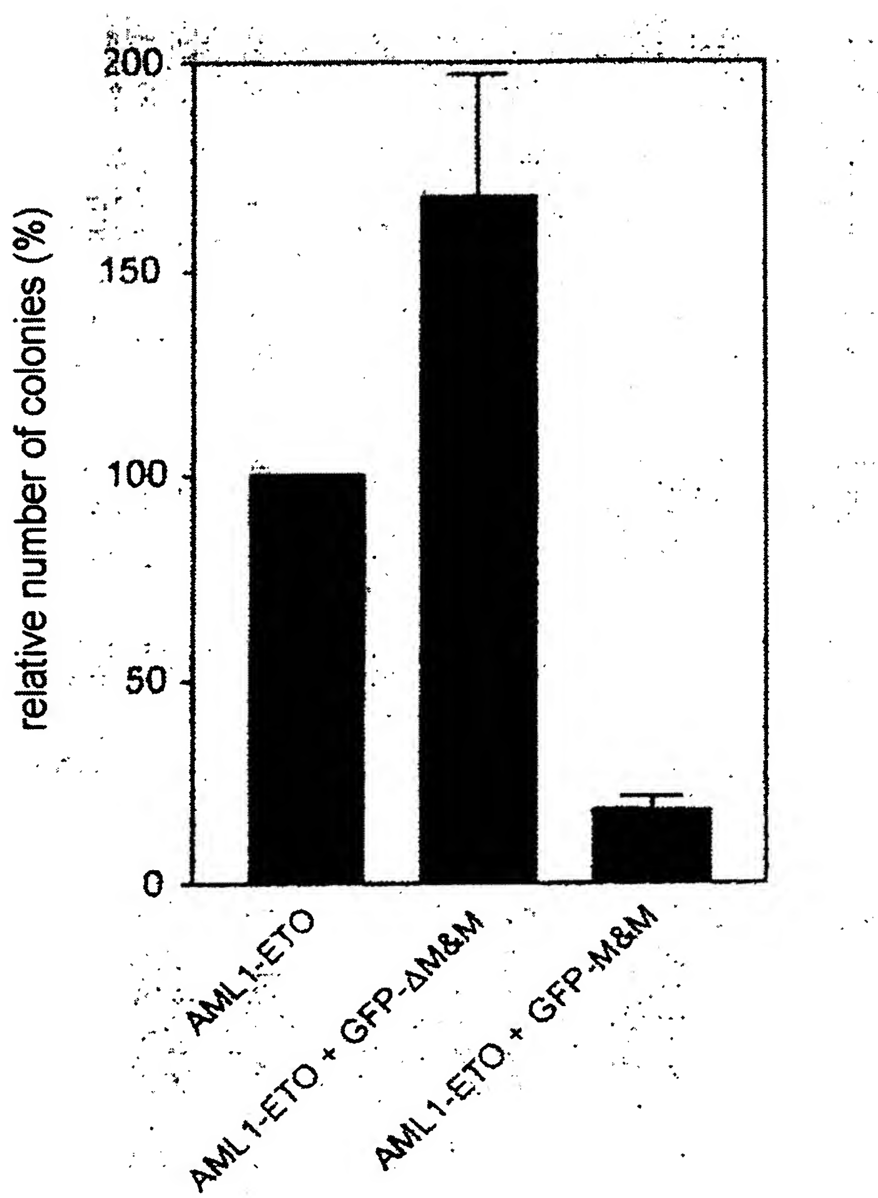

relative number of colonies (%)
200
150
100
50
0
AML1-ETO
AML1-ETO + GFP-ΔM&M
AML1-ETO + GFP-M&M

FIGURE 5D

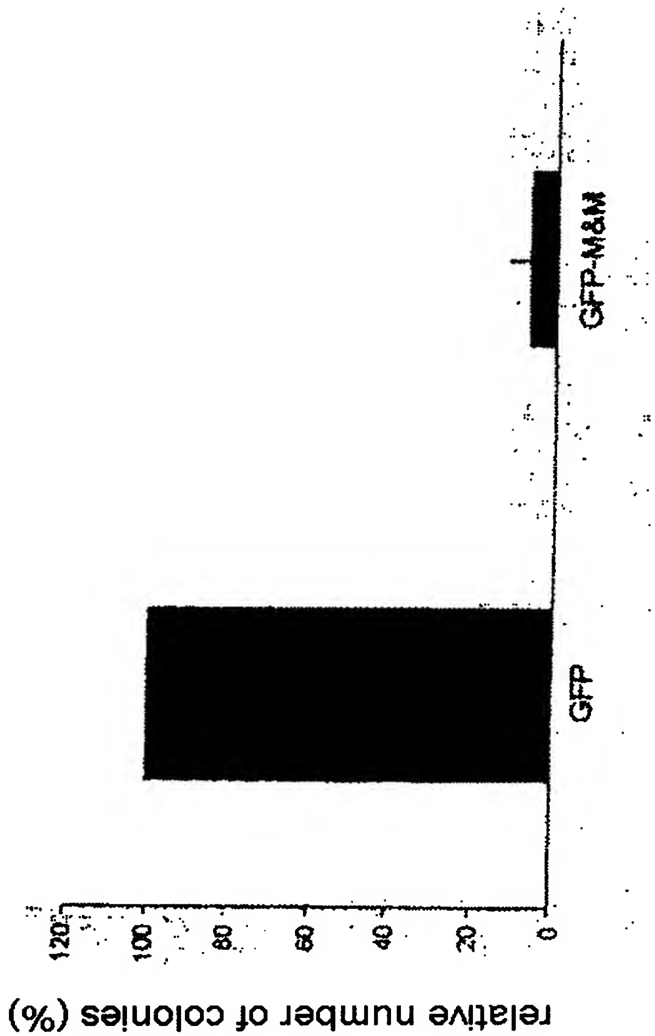


FIGURE 6A

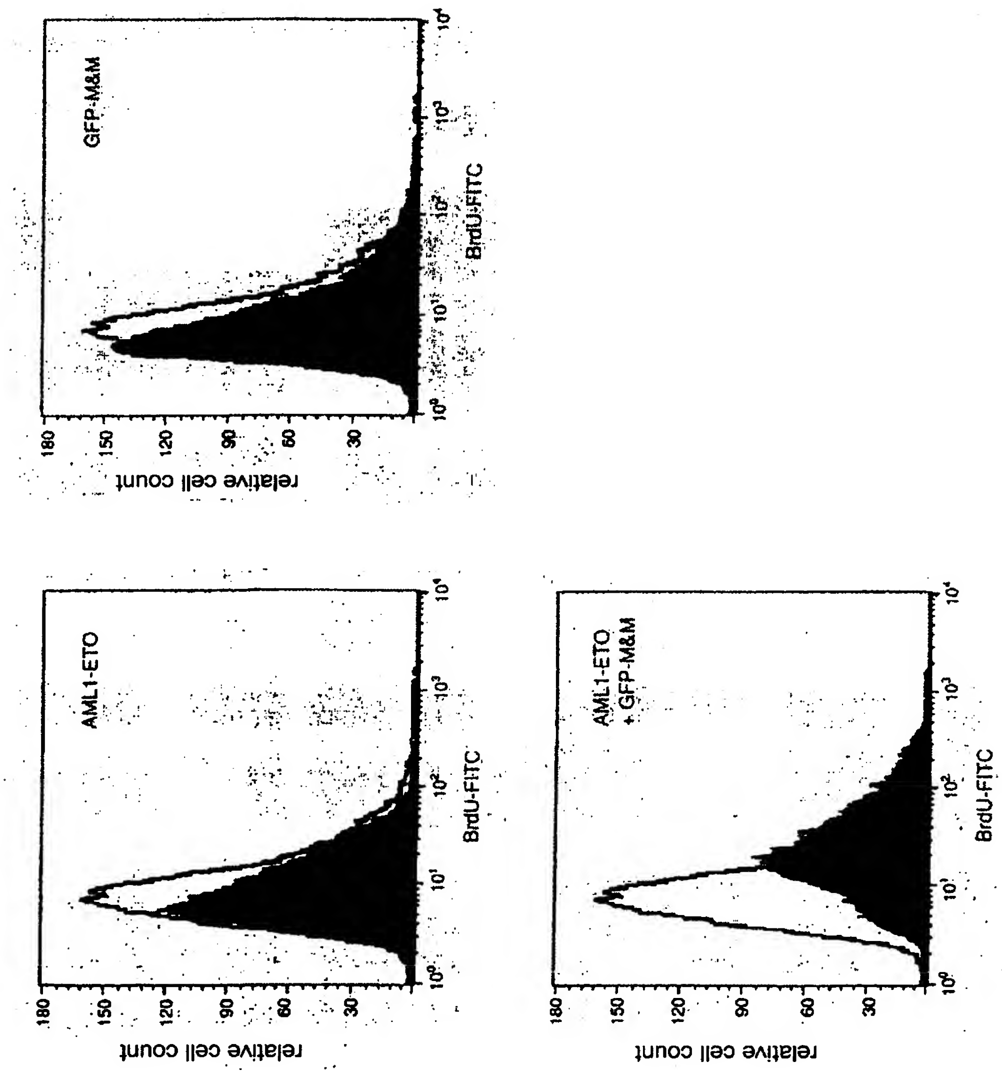
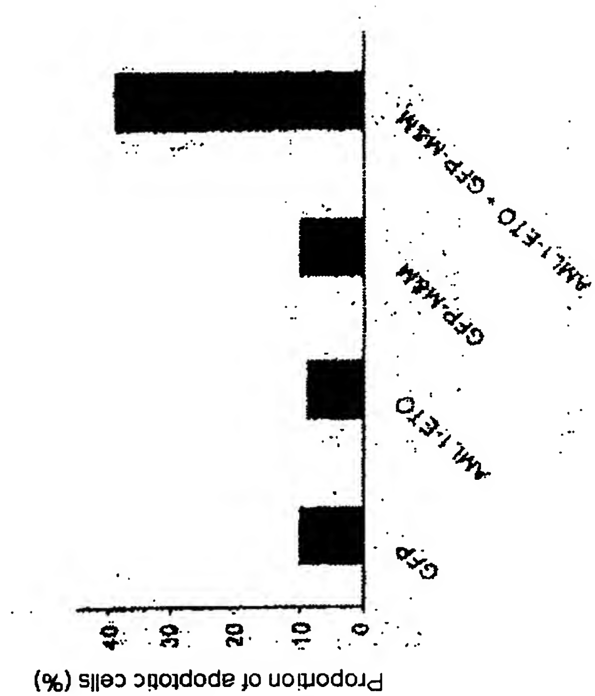


FIGURE 6B


Proportion of apoptotic cells (%)
0
10
20
30
40
GFP
AML1-ETO
GFP-M4M
AML1-ETO + GFP-M4M